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Glyphosate Stimulates the Accumulation of N-Compounds, Grain Yield and Seed Vigor in Glyphosate-Resistant Soybean

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Abstract

Glyphosate-resistant (GR) soybean is widely used in agriculture, however, plants exposed to herbicide show physiological changes. This study investigated the effect of treatments with glyphosate on the metabolism of N compounds, crop yield and physiological quality of seeds in GR soybean. The plants were grown in field experiment, located in the city of Selvíria, MS, Brazil. Glyphosate was applied postemergence at V_4 crop stage in a dose-response, including four rates (0; 360; 720 and 1440 g e. a. ha^{-1}) with four replicates. Crop yield, physiological and biochemical features were determined. The results revealed previously unreported stimulant effects of glyphosate on GR soybean plants. Glyphosate changed the ureide shape in leaves, but did not change the concentration of total ureides, indicating maintenance of biological nitrogen fixation in plants exposed to herbicide. Amino acids concentration increased in plants submitted to higher doses. GR soybean showed higher crop yield and seed vigor with increased glyphosate doses. The results of this study indicate that glyphosate does not cause stress to the plant; however, it modulates a distinct response in plant development due to the protective gene inserted. This study can serve as a matrix for additional studies in order to seek clarification of responses of resistant/tolerant plants to glyphosate.

Keywords: plant development, transgenic soybean, herbicide, nitrogen

1. Introduction

Soybean (*Glycine max* (L.) Merrill) stand out as one of the world's main commodities, with the United States being the largest producer nations, followed by Brazil and Argentina (Krenchinsk et al., 2017). Much of the success of soybean cultivation worldwide occurs due to the development of transgenic cultivars, with about 93% of soybean cultivars in Brazil being transgenic (Clive, 2014).

Soybean cultivars with the Roundup Ready® gene was developed through genetic engineering, aiming at improvements in integrated management of weeds. Thus, after spraying crop with the herbicide, only the weeds are affected, while the lethal effect is absent in GR soybean. The gene inserted is from the soil bacterium *Agrobacterium tumefaciens* strain CP4, which encodes a variant enzyme of EPSPs, CP4-EPSPs, conferring GR soybean resistance to glyphosate (Franz et al., 1997).

Although GR soybean displays distinct resistance to glyphosate, little is known about its effect on physiological processes and plant development, since that there are other modes of action of glyphosate, not only specific inhibition of EPSPs (Franz et al., 1997; Reddy et al., 2004). Literature data show that glyphosate causes visual injury symptoms on GR soybean plants (Zobiole et al., 2012), altering the nutritional status, mainly, with symptoms of Zn, Fe and Mn deficiency (Johal & Huber, 2009), reduction of photosynthetic efficiency and water use (Zobiole, 2010a, 2010b). However, in some non-transgenic plants subjected to low glyphosate doses, the stimulant effect is frequently observed (Cedergreen, 2008; Dalley & Richard, 2010).

As glyphosate is widely used in crops (Duke et al., 2008) and the distinct resistance to glyphosate in GR soybean causes physiological changes (Zobiole et al., 2010a, 2010b), the hypothesis of this work is that GR soybean

plants under treatments with glyphosate show the physiological and biochemical characteristics alterated, culminating with change in crop yield. Thus, the objective of the present study was to investigate the effects of treatments with glyphosate on the metabolism of N compounds, crop yield and physiological quality of seeds in GR soybean.

2. Method

2.1 Study Site and Climate Data

The experiment was conducted in the 2015/2016 season in an experimental localizated in Selvíria, Mato Grosso do Sul, Brazil (55°22′ W and 22°20′ S, 335 m). According to the USDA classification, the soil is Rhodic Hapludox (Soil Survey Staff, 2010). Before the installation of the experiment, soil samples composed of 20 sub-samples, at 0.0-0.2 m layer, were collected to determine the chemical characteristics, in accordance with Raij et al. (1997): organic matter 22 g dm^{-3}; pH (CaCl2) 5.1; P (resin) 20 mg dm^{-3}; K 2.6 mmolc dm^{-3}; Ca 17 mmolc dm^{-3}; Mg 12 mmolc dm^{-3} and bases saturation of 51%. The climate is type Aw (Köppen classification) with annual precipitation average 1370 mm. The average annual temperature is 23.5 °C and relative air humidity 66% (annual average). During the experiment period, the average minimum and maximum temperatures were 21.9±1.5 °C and 33.1±2.2 °C, respectively. Additionally, periodic assessments of temperature and precipitation data in the experimental area were made. The data were obtained from a meteorological station of the experimental farm, located at 180 m of the experimental area (Figure 1).

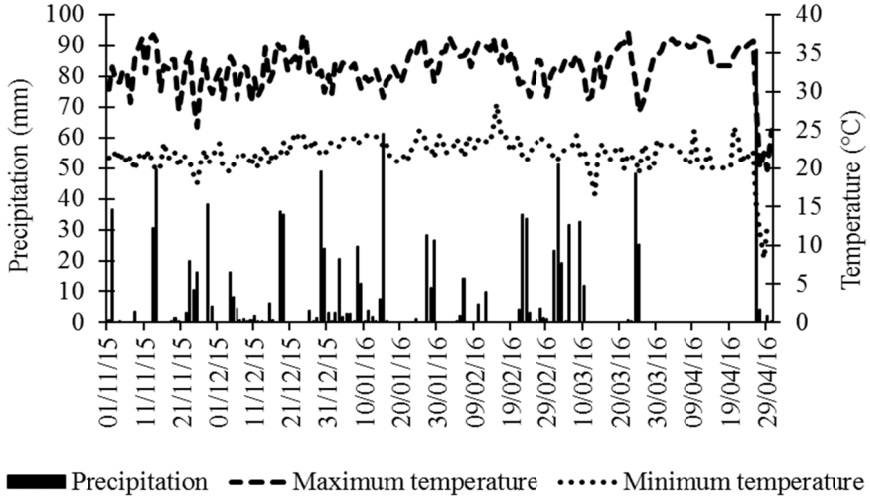

Figure 1. Precipitation (mm), maximum temperature (°C) and minimum temperature (°C) during the experimental in 2015/2016 season agricultural crop

2.2 Plant Cultivation

Initially, the seeds were treated with a product containing carboxym+thiram, at a dose of 50 + 50 g (active ingredient) 100 kg $seeds^{-1}$. After drying the product, the application was made with peat-base inoculant aiming to achieve a minimum of 1.2×10^6 colony-forming units of *Bradyrhizobium* per seed, as recommended for tropical regions (Hungria et al., 2017).

The fertilization for sowing was performed according to soil analysis to obtain high yield using 300 kg ha^{-1} of 04-20-20 formulation applied in the furrow seeding, according to Raij et al. (1997). On Nov. 16, 2015, mechanical seeding was carried out jointly with fertilizing, using the soybean cultivar BMX Potência RR in an area previously plowed and harrowed. The spacing between rows was 0.45 m and a density was 15 seeds per m with a population of 300,000 plants ha^{-1} (90% germination). The emergence of seedlings occurred on 21 Nov 2015. The experimental plots were set up by seven rows of 10 m and the three central rows were considered useful area, disregarding 1 m at both ends (10.8 m²).

The recommended dose of herbicide of 720 g ha^{-1} (e. a. – equivalent acid) (Ludwig et al., 2011) was considered and on Dec. 14, 2016, at phenological stage V_4 (Fehr et al., 1971), glyphosate was applied as follows: T_0 – Control (0 g e. a. ha^{-1}); T_1 – underdose recommended (360 g e. a. ha^{-1}); T_2 – recommended dose (720 g e. a. ha^{-1})

and T_3 – overdose (1440 g e. a. ha^{-1}). The application of the herbicide was held with a costal sprayer (manual application) with volume of 200 L ha^{-1}. The source used was the herbicide Roundup® (trade name), whose formulation corresponds to composition: salt of isopropylamine N-(phosphonomethyl) glycine 480 g L^{-1} (48% m v^{-1}), equivalent acid of N-(phosphonomethyl) glycine (glyphosate) 360 g L^{-1} (36% m v^{-1}), and inert ingredients of 684 g L^{-1} (68% m v^{-1}). The supplemental mechanical (manual) weed control was performed during the entire crop cycle, especially in the plots of the control treatment that was used only the manual weed control method.

2.3 Extraction of N Compounds for Quantification and Qualitative Analysis of Amino Acids

On Jan. 18, 2016, at the phenological phase R_2 (Fehr et al., 1971), the first newly expanded leaf (from the main stem) was collected and later the samples were placed in plastic bags. Next, the plant material was taken to the lab and placed in a freezer for further analysis.

N compounds were extracted according to recommendations of Bielski and Turner (1966).

For 1 g of fresh material, 10 mL of MCW solution (60% mL Methanol, 25% mL Chloroform, 15% mL H_2O) was added. The material was mashed and then centrifuged. After centrifugation, 1 mL of chloroform + 1.5 mL of H_2O was added for each 4 mL supernatant. After 24 h under refrigeration for to phase separation, the water-soluble phase was used for the analysis of ureides, amino acids and ammonia.

It was added 10 mL of NaOH 0.1 N to the precipitate, which was subsequently homogenized and, after centrifugation, the supernatant was used for the analysis of total protein.

The extraction was performed in plants entirely dependent on N biological fixation for nutrient supply throughout the plant cycle.

2.3.1 Quantitative Analysis of Total Ureides (Allantoin and Allantoic Acid)

The Vogels and Van der Drift (1970) method was used to quantify changes in the concentration of ureides (allantoin and allantoic acid) in the leaf tissue. The method determined the amount of glyoxylate formed after hydrolysis from the reaction with potassium Ferritian and phenylhydrazinium. Allantoin was used as a quantification standard.

2.3.2 Quantitative Analysis of Total Soluble Amino Acids

The Yemm and Cocking (1955) method was used to quantify changes in the concentration of total soluble amino acids in the leaf tissue. Leucine was used as a quantification standard.

2.3.3 Quantitative Analysis of Ammonia

The McCullough (1967) method was used to quantify changes in the concentration of soluble ammonia in the leaf tissue. Leucine was used as a quantification standard.

2.3.4 Quantitative Analysis of Total Protein

The Bradford (1976) method was used to quantify changes in the concentration of proteins in the leaf tissue. BSA was used as a quantification standard.

2.4 Quantification of Production Components

On Apr. 6, 2016, ten plants were collected manually in a randomized manner from one of the rows in the useful area of the plot. The plant material was placed in bags and taken to a concrete yard for final drying. Subsequently, impurities were eliminated and manual measurements of the number of pods per plant, number of grains per pod, number of grains per plant and mass of 25 seeds were made.

2.5 Estimation of Grain Yield

On Apr. 6, 2016, plants in two rows of the useful area of the plot were harvested manually. The plants were placed in bags and taken to a concrete yard for final drying. Subsequently, the threshing and cleaning of the material were carried out in a stationary threshing machine. The grain samples obtained were placed in paper bags and the grain mass of each sample was measured with a precision scale. Afterward, moisture was determined (greenhouse method – 105±3 °C/24 h) for subsequent correction of grain mass to 13% humidity. Grain yield was estimated in kg ha^{-1}.

2.6 Physiological Analysis of Seeds

The seeds obtained to quantify production components were used to perform all the physiological analysis of seeds.

2.6.1 First Germination Count, Total Germination and Germination Speed Index

The germination test was performed with 50 seeds per treatment, containing four repetitions each. The seeds were sown on germination paper moistened with distilled water at a ratio 3-fold the weight of the dry paper. The seeds were then incubated in germination chambers, at constant temperature of 25 °C. The counts were performed at 5 and 9 days after the test installation and the results were expressed as percentage of normal seedlings (Brasil, 2009). The test of germination speed index was determined according to the equation proposed by Krzyzanowski et al. (1999).

2.6.2 Dry Matter Mass of Seedlings

After the germination test, 10 seedlings for each repeat treatment were collected to determine the dry matter mass. The seedlings were taken to a forced ventilation oven at 80 °C for a period of 24 h (Krzyzanowski et al., 1999). After reaching constant weight, the material was weighed on an analytical scale.

2.6.3 Electric Conductivity

The test was conducted in accordance with the methodology proposed by Loeffler et al. (1988). Four replicates of 50 seeds were used, weighed and immersed in 75 mL of distilled water inside plastic cups at 25 °C. After soaking for 24 h, the reading of the electric conductivity of the solution was determined with a Digimed CD-20.

2.6.4 Accelerated Aging

Seeds were placed in plastic gerbox boxes, functioning as the mini-cameras and containing 40 mL of water at the bottom, which were kept at 41 °C for 60 h. Afterward, four repetitions of 50 seeds were place to germinate following the method described above. The evaluation was carried out on the 4th day after sowing, according to Brasil (2009).

2.7 Statistical Analysis

The experimental design was a randomized block design with four treatments and four replications. The data were subjected to analysis of variance and compared by regression test at 5% probability using the software SISVAR®

3. Results

Glyphosate did not alter the concentration of total protein, total ureides, allantoin and soluble ammonia in the leaves in any of the treatments used in GR soybeans ($p > 0.05$) (Table 1). However, the concentration of total soluble amino acids in the leaves responded positively ($p < 0.01$) in treated plants with higher doses of the herbicide (Figure 2). Glyphosate did not change total ureide accumulation in the leaves ($p > 0.05$). However, there was a change in the ureide shape in the foliar tissue, caused by the reduction in the allantoic acid concentration in leaves of plants subject to treatment with the underdose and recommended dose of herbicide ($p < 0.05$) (360 and 720 g e. a. ha^{-1}, respectively). This condition reverted when plants were exposed to overdose of the herbicide (Figure 2).

Table 1. Protein contents (μmol g^{-1} FW), total ureides (μmol g^{-1} FW), allantoin (μmol g^{-1} FW) and ammonia (μmol g^{-1} FW) in leaves of GR soybean subjected to different treatments with glyphosate

Treatment with glyphosate	Protein [(1)]	Total ureides [(1)]	Allantoin [(1)]	Ammonium [(1)]
---------- g e. a. ha^{-1} ----------	---------- μmol g^{-1} FW ----------			
0	0.92	5127.35	2126.55	2.48
360	1.50	3643.68	1827.76	2.46
720	0.69	4398.05	2442.19	2.10
1440	0.89	5995.90	2347.42	2.62
C.V. (%)	32.60	31.22	32.25	17.84

Note. Control (0 g e. a. ha^{-1}); Glyphosate underdose recommended (360 g e. a. ha^{-1}); Glyphosate recommended dose (720 g e. a. ha^{-1}); glyphosate overdose (1440 g e. a. ha^{-1}); [(1)] Not significant in the F test at 5% probability; C.V. (%) Coefficient of variation; Treatment with base in the equivalent acid of glyphosate (e. a.); n = 16.

There was no change in the number of pods per plant, grains per pod, grains per plant and mass of 25 seeds in any of the treatments ($p > 0.05$) (Table 2). However, the grain yield of GR soybean was influenced positively

with the increase of the glyphosate concentration ($p < 0.01$), showing a better grain yield of 44%, 39% and 63% when exposed to concentrations of 360, 720 and 1440 g e. a. ha^{-1}, respectively (Figure 3).

Table 2. Number of pods per plant, number of grains per pod, number of grains per plant and mass of 25 seeds (g) in GR soybean plants subjected to different treatments with glyphosate

Treatment with glyphosate	Number of pods per plant (1)	Number of grain per pod (1)	Number of grain per plant (1)	Mass of 25 seeds (1)
---------- g e. a. ha^{-1} ----------				--------- g ----------
0	79.85	1.71	138.50	3.49
360	67.68	2.14	132.05	3.55
720	66.35	1.81	118.43	3.75
1440	108.35	1.36	108.73	3.74
C.V. (%)	34.13	33.78	30.47	3.73

Note. Control (0 g e. a. ha^{-1}); Glyphosate underdose recommended (360 g e. a. ha^{-1}); Glyphosate recommended dose (720 g e. a. ha^{-1}); glyphosate overdose (1440 g e. a. ha^{-1}); (1) Not significant in the F test at 5% probability; C.V. (%) Coefficient of variation; Treatment with base in the equivalent acid of glyphosate (e. a.); n = 16.

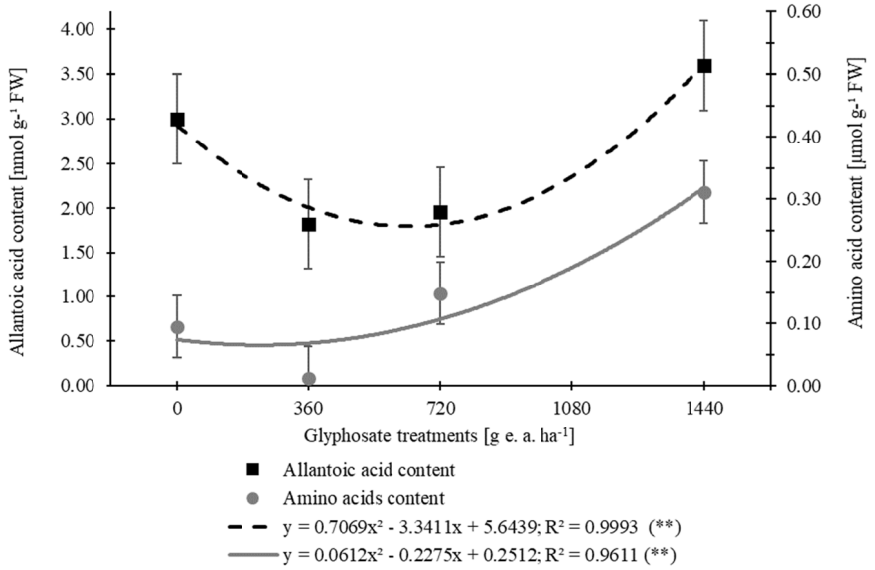

Figure 2. Allantoic acid concentration ($p < 0.05$) and soluble amino acid ($p < 0.01$) in leaves of GR soybeans subject to different glyphosate doses. (**) Regressions significant at $p < 0.01$; n = 16

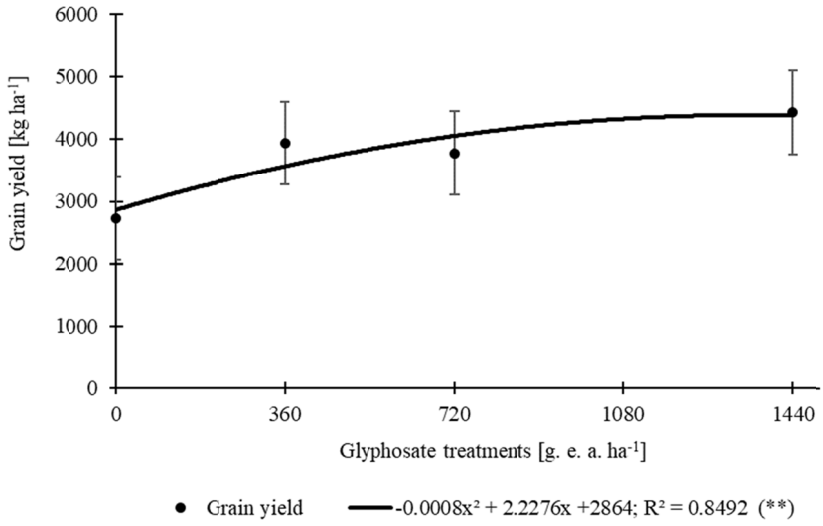

Figure 3. Grain yield GR soybeans due to the increase in glyphosate dose used ($p < 0.01$). (**) Regression significant at $p < 0.01$; $n = 16$

Regarding the effects on the physiological quality of seeds, glyphosate did not alter the first germination count, total germination, germination speed index and dry matter mass of seedlings in any of the treatments ($p > 0.05$) (Table 3). On the other hand, in some physiological vigor analyses, seeds performed better, as observed on electrical conductivity of seeds that was reduced with the increase in herbicide concentration ($p < 0.01$) (Figure 4). Positive effects were also recorded in the accelerated aging test, with increased tolerance to damages caused by high temperature and humidity of 15%, 24% and 3% at concentrations of 360, 720 and 1440 g e. a. ha^{-1}, respectively ($p < 0.05$) (Figure 4).

Table 3. First germination count (%), total germination (%), germination speed index and seedling dry mass (mg) of seeds obtained from plants treated with different glyphosate doses

Treatment with glyphosate	First germination count [(1)]	Total germination [(1)]	Germination speed index [(1)]	Seedling dry mass [(1)]
---------- g e. a. ha^{-1} ----------	---------- % ----------			--------- mg ---------
0	71.00	71.00	7.10	30.98
360	78.50	78.50	7.85	26.11
720	64.00	64.00	6.40	22.59
1440	71.50	71.50	7.15	25.88
C.V. (%)	14.05	14.05	14.05	34.81

Note. Control (0 g e. a. ha^{-1}); Glyphosate underdose recommended (360 g e. a. ha^{-1}); Glyphosate recommended dose (720 g e. a. ha^{-1}); glyphosate overdose (1440 g e. a. ha^{-1}); [(1)] Not significant in the F test at 5% probability; C.V. (%) Coefficient of variation; Treatment with base in the equivalent acid of glyphosate (e. a.); $n = 16$.

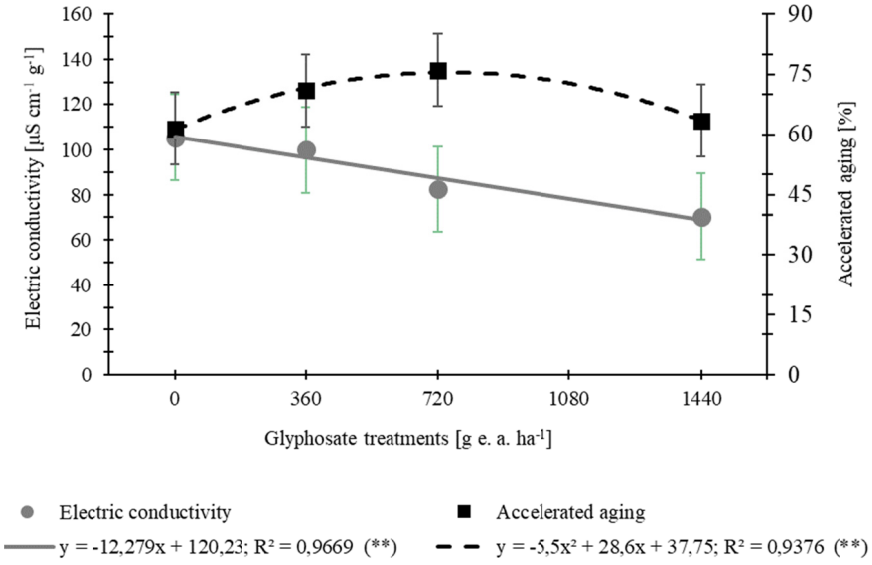

Figure 4. Electric conductivity ($p < 0.01$) and accelerated aging of seeds ($p < 0.05$) obtained from plants treated with different glyphosate doses. (**) Regressions significant at $p < 0.01$; $n = 16$

4. Discussion

This study highlights the importance of elucidating responses of a genetically modified plant, aiming to withstand exposure to glyphosate in an ecophysiological context. Previous studies from our research group showed that glyphosate moderately affected N fixation and assimilation, but there were no drastic metabolic changes for the amino acids, proteins, chlorophylls and ureides in vegetative stage of GR soybean (Bomfim et al., 2017); however, no data are available on the effects of glyphosate on the content of N compounds during the reproductive stage. As for qualitative variation in the form of ureides (Figure 2), there is no clear explanation in the literature, although some studies have reported this response depending on the N source in the medium (Camargos et al., 2009; Camargos & Sodek, 2010). In this study, no changes were observed in concentrations of some N compounds in the leaves (Table 1), but rather an increased concentration of amino acids (Figure 2). Even with the qualitative change in the ureide shape in the leaves, the concentration of total ureides was not affected (Table 1).

Ureides are responsible for most N transported via the xylem to the shoot in soybean plants and are, therefore, used as signals of efficiency of the biological fixation of N (Herridge & Peoples, 1990). This shows that the optimal levels of N-fixation were kept, even in plants exposed to glyphosate, which are contrasting to studies that showed negative effects of glyphosate on fixation and nodulation in GR soybeans (Reddy & Zablotowicz, 2003; Zobiole et al., 2011).

Due to the distinct resistance incorporated in GR soybean, the plant exposed to the herbicide displays resilience mechanisms to potentially harmful agent, not exempting effects on important in the processes of plant development. Transgenesis promotes an alternative route that aims to preserve the shikimic acid pathway, which is inhibited by specific action of glyphosate on the EPSPs enzyme, providing soybeans resistance to glyphosate (Zablotowicz & Reddy, 2007). However, the literature reports that herbicides may alter the way the photoassimilates are allocated to different plant organs, due to its effect on plant development (Cedergreen et al., 2007).

Full flowering coincides with the peak of photoassimilates accumulation in the leaves, because the leaves are a strong drain until entrance of the vegetables into the reproductive stage (Soudry et al., 2005). Therefore, the higher status of N-compounds in the leaves (Figure 2) are possibly related to the effect of herbicides on plant development (Cedergreen et al., 2007).

In order to protect the species in environments, in response to herbicide, senescence processes were anticipated in plants exposed to glyphosate, which subsidizes the greater grain yield in herbicide-treated plants (Figure 3). The modifications mentioned in the plant development pattern resemble the hormesis effect, as described by

Calabrese and Baldwin (2008) that, under low phytotoxicity conditions, plants present responses that stimulate the development (Belz et al., 2010).

By comparison, the use of low glyphosate doses in sugar cane is a consecrated practice as a measure to accelerate the maturation process (Dalley & Richard, 2010). Although these data refer to the non-transgenic plant, from the ecophysiology viewpoint, vegetables tend to accelerate their development under low phytotoxicity conditions (Calabrese & Baldwin, 2008), aimed at the maintenance of specie as a strategy of phytogeographic perpetuation (Cedergreen et al., 2007).

In experiment using low glyphosate concentration, the hormesis effect is often seen in plants (Cedergreen, 2008; Dalley & Richard, 2010); however, because GR soybean plants present the protective gene to glyphosate, AroA (Zablotowicz & Reddy, 2007), the hormesis effect is observed at higher concentrations. With the process of accelerated monocarpic senescence, photoassimilates are distributed to high-demand sites, while they subsidize the positive effect on physiological vigor of seeds of plants treated, as observed in the electrical conductivity and accelerated ageing tests (Figure 4).

The electrical conductivity test indicates the integrity level of cell membranes due to the leachate quantity in the soaking solution, and the seed vigor is inversely proportional to the reading of electric conductivity (Hepburn et al., 1984; Marcos-Filho et al., 2001). The relative earliness of monocarpic senescence in treated plants caused the acceleration of photoassimilates distribution of source organs for the seed, and the reserve of seed is formed mainly by proteins and lipids (Bellaloui et al., 2015). The larger stocks of these substances in seeds resulted in the proper structuring of cell membranes, justified by the fact that proteins and lipids are essential to the integrity of biological membranes (Nelson & Cox, 2014).

In line with the data on electric conductivity (Figure 4), seeds from treated plants showed better performance in the accelerated aging test (Figure 4), which confers seed tolerance to adverse conditions of high temperature and humidity (Marcos-Filho et al., 2001). The better performance of seeds of treated plants is attributed to a direct relationship between the amount of recently stocked reserve and physiological quality of seeds (Pereira et al., 2015), since glyphosate stimulated plant development in order to accumulate more reserve stock in seeds.

GR soybean seems to respond differently to the glyphosate action, not necessarily characterizing negative stress to the plant, but modulating one response to distinct plant development due to the protective gene inserted. Therefore, stimulant effect of glyphosate on GR soybean plants is clear. Evidences exist and are categorical, but new experiments at field level will be necessary to detail that process. Thus, the glyphosate may be applicated not only by control weed, but also by stimulate the GR soybean development.

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